

ORIGINAL ARTICLE

# Integrin alpha 10, CD44, PTEN, cadherin-11 and lactoferrin expressions are potential biomarkers for selecting patients in need of central nervous system prophylaxis in diffuse large B-cell lymphoma

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## Abstract

Central nervous system (CNS) relapse is a devastating complication that occurs in about 5% of diffuse large B-cell lymphoma (DLBCL) patients. Currently, there are no predictive biological markers. We wanted to study potential biomarkers of CNS tropism that play a role in adhesion, migration and/or in the regulation of inflammatory responses. The expression levels of ITGA10, CD44, PTEN, cadherin-11, CDH12, N-cadherin, P-cadherin, lactoferrin and E-cadherin were studied with IHC and IEM. GEP was performed to see whether found expressional changes are regulated at DNA/RNA level. IHC included 96 samples of primary CNS lymphoma (PCNSL), secondary CNS lymphoma (sCNSL) and systemic DLBCL (sDLBCL). IEM included two PCNSL, one sCNSL, one sDLBCL and one reactive lymph node samples. GEP was performed on two DLBCL samples, one with and one without CNS relapse. CNS disease was associated with enhanced expression of cytoplasmic and membranous ITGA10 and nuclear PTEN ( $P < 0.0005$ ,  $P = 0.002$ ,  $P = 0.024$ , respectively). sCNSL presented decreased membranous CD44 and nuclear and cytoplasmic cadherin-11 expressions ( $P = 0.001$ ,  $P = 0.006$ ,  $P = 0.048$ , respectively). In PCNSL lactoferrin expression was upregulated ( $P < 0.0005$ ). IEM results were mainly supportive of the IHC results. In GEP CD44, cadherin-11, lactoferrin and E-cadherin were under-expressed in CNS disease. Our results are in line with previous studies, where gene expressions in extracellular matrix and adhesion-related pathways are altered in CNS lymphoma. This study gives new information on the DLBCL CNS tropism. If further verified, these markers might become useful in predicting CNS relapses.

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## Abbreviations

CNS	central nervous system
DLBCL	diffuse large B-cell lymphoma
EMT	epithelial-mesenchymal transition
GC	germinal centre
GEP	gene-expression profiling
IHC	immunohistochemistry
IEM	immunoelectron microscopy
PBS	phosphate-buffered saline
PCNSL	primary CNS lymphoma
PTEN	phosphatase and tensin homolog
sCNSL	secondary CNS lymphoma
sDLBCL	systemic DLBCL

## Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma subtype, representing about 30% of all lymphomas. In addition to systemic disease, it can also manifest in the central nervous system (CNS) in the form of secondary CNS involvement of systemic DLBCL or as primary CNS lymphoma (PCNSL). The latter is a rare disease, representing 1–2% of all non-Hodgkin lymphomas (1).

Of systemic DLBCL patients, around 5% will have disease involvement in the CNS either during diagnosis, or more commonly, a few months afterwards (2,3). The inclusion of rituximab in the treatment has increased the effective control of the systemic DLBCL, but the data considering its effect on the risk of a CNS relapse is contradictory. Before the rituximab era, CNS involvement was usually seen simultaneously with refractory, widespread systemic disease (4,5). This paradigm has changed and currently the emergence of solitary CNS relapses has become an increasing problem. Patients with a CNS relapse have a dismal prognosis. High-dose methotrexate and cytarabine included in the primary treatment of systemic DLBCL seem to reduce the risk of a CNS relapse. However it is associated with considerable toxicity and the identification of patients at high risk of CNS relapse still remains problematic today (6,7).

Currently the patient selection for CNS prophylaxis is carried out by clinical risk assessment based on the following clinical factors: high International Prognostic Index (IPI) score, advanced stage, elevated lactate dehydrogenase, age > 60 years and involvement of extranodal sites (such as paraspinal or paranasal location, testes or breast). At best, this clinical evaluation is able to identify a patient population with a 20% risk of CNS disease, thus the number needed to treat to prevent one relapse is high. There is also a small group of patients who will later develop CNS disease and fail to be detected by this clinical risk score. There is an unmet clinical need for biological markers that could be used for predicting CNS recurrence (8).

Based on our previous study and literature, we hypothesized that CXCR4/CXCL12 signalling mediates the CNS homing of lymphoma cells and provokes an epithelial-mesenchymal transition (EMT)-like process (9). CXCR4/CXCL12 signalling has been linked to the expression and/or activity of integrins, CD44 (a cell-surface glycoprotein), phosphatase and tensin homolog (PTEN), cadherin-11, N-cadherin and E-cadherin (10–14). Integrin alpha 10 (ITGA10), CD44, PTEN, cadherin-11, cadherin-12 (CDH12), N-cadherin and E-cadherin have also been linked to EMT and associate with the expression of EMT transcription factors (15–21). Lactoferrin levels have been reported to be elevated in the cerebrospinal fluid of patients with CNS involvement of leukemia and lymphoma (22). In addition lactoferrin was of interest as it has been shown to downregulate the expression

of intercellular adhesion molecule-1 (ICAM-1), this way possibly reducing the recruitment of leukocytes to the inflammatory site (23). In this work, we performed a screening of the above-mentioned molecules with an aim of finding possible biomarkers in order to identify patients with a high risk of CNS recurrence. The expression levels of these molecules were studied by means of immunohistochemistry (IHC) and immunoelectron microscopy (IEM). Gene-expression profiling (GEP) was performed to identify whether the expressional changes seen are regulated at gene-expression level.

## Materials and methods

### Patients and samples

Patients included in the study were diagnosed and treated at the University Hospitals of Oulu, Jyväskylä, Kuopio and Tampere between 1997 and 2013. The median age of patients at diagnosis was 65 (range 27–82). The control group was selected from patients who did not have any signs of CNS disease during diagnosis, therapy or follow-up from the time of diagnosis until the end of the year 2015. Also the control group did not receive CNS prophylaxis. Patient information was collected from the hospital records, blinded from the laboratory results and used as encrypted data. Clinical data was collected from patients with primary systemic disease, including GC/non-GC phenotype, age, lactate dehydrogenase, IPI and the number of extranodal lesions. Permission to use patient samples included in the study was granted by the Finnish National Supervisory Authority for Welfare and Health (6622/05.01.00.06/2010). This study was approved by the Ethics Committee of Oulu University Hospital (42/2010, 23.6.2010).

The study material for IHC consisted of diagnostic biopsy samples from 96 patients with newly diagnosed DLBCL, including 15 patients with PCNSLs, 31 with secondary CNS lymphomas (sCNSLs) and a control group of 50 patients with systemic DLBCLs (sDLBCL) that did not receive CNS prophylaxis. The sCNSL samples were diagnostic lymph node samples from primary diagnosis. IEM included two PCNSL samples, one sCNSL (brain biopsy), one sDLBCL and one reactive lymph node sample. GEP included two diagnostic lymph node fresh frozen tissue samples, one sDLBCL and one sCNSL sample from primary diagnosis.

### Immunohistochemistry

Slide preparation was performed as described earlier except that before deparaffinization the slides were incubated overnight at +37°C (24). The antigen retrieval and the primary antibodies and their concentrations are presented in Supplementary Table 1, available at *Carcinogenesis* Online. Sample preparation continued as described in our previous study (9). To determine the germinal centre (GC) phenotype of each lymphoma sample, Hans' algorithm was used with the same antibodies as in our previous study (9,25).

### Immunoelectron microscopy

Tissue pieces were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with 2.5% sucrose for 6 h at RT then immersed and stored in 2.3 M sucrose in PBS. Tissue pieces were placed on specimen stubs and frozen in liquid nitrogen. Ultrathin sections (70 nm) were cut with a Leica EM UC7 cryo-ultramicrotome. For immunolabelling, ultrathin sections were incubated in 0.1 % glycine + PBS followed by incubation in 1% bovine serum albumin in PBS. All washes and antibody dilutions were performed in PBS + 1% bovine serum albumin. After incubation with the primary antibodies the sections were incubated with Protein A-gold conjugate (10 nm). Rabbit anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, UK) was used as a secondary antibody. Sections were embedded in methylcellulose and examined in a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, the Netherlands) equipped with a Quemesa digital camera (EMSIS GmbH, Münster, Germany). The antibodies used were the same as for IHC.

### Gene-expression profiling

The RNA isolation was performed using the RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. A GeneChip protocol



was used and the experimental procedures were performed in accordance with the Affymetrix GeneChip Expression Analysis Technical Manual. Using 5 µg of total RNA as template, a double-stranded DNA was synthesized by means of the One-cycle cDNA synthesis kit (Affymetrix, USA) and T7-(dT)24 primer. GeneChip Sample Cleanup Module (Qiagen, USA) was used to purify the DNA. By using an *in vitro* transcription (IVT) labelling kit (Affymetrix, USA) according to the manufacturer's instructions, the IVT was performed to produce biotin labelled complementary RNA. Biotinylated complementary RNA was cleaned using the GeneChip Sample Cleanup Module (Qiagen), fragmented to 35–200 nucleotides, and hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays that contain ~55000 human transcripts. After washing, the array was stained with streptavidin-phycoerythrin (Molecular Probes). The staining signal was amplified by biotinylated anti-streptavidin (Vector Laboratories) followed by second staining with streptavidin-phycoerythrin, and then scanned on the GeneChip Scanner 3000.

## Analysis

In the analysis of IHC staining, the location of positivity in the malignant B-cells was determined separately in the nucleus, cytoplasm and/or cell membrane. Then the percentage of positive cells was estimated separately for each location, from 0 to 100% with 5% accuracy. Micrographs from IHC staining patterns were obtained using an Olympus DP11 digital microscope camera (Olympus, Center Valley) and an Olympus BX41 microscope. To import micrographs an HP Photo and Imaging software package (Hewlett-Packard Company, Palo Alto) was used.

The IEM samples were reviewed using a transmission electron microscope. A general assessment was made of the expression pattern, taking into account the location (nucleus, cytoplasm and/or cell membrane) and the amount of positivity detected. Micrographs of each sample were taken using a Quemesa digital camera (EMSIS GmbH, Münster, Germany) and a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, the Netherlands) as part of the analysis.

The expression data gained from GEP was analysed using dChip software (26).

For statistical analysis, IBM SPSS Statistics for Windows 20.0 software (IBM Corp. Released 2011. Armonk, NY) was used. In order to analyse the associations between diagnosis (sDLBCL versus sCNSL) and the IHC protein expressions, the cut-off points for the latter were chosen using the receiver operating characteristic curve. The area under the curve (AUC) shows sensitivity versus 1-specificity. Only values greater than 0.5 were taken into account, values closer to 1.0 possessing a better discriminatory power. Due to the small study material, AUCs were relatively low. Cut-off points were chosen by estimating a good balance between sensitivity and specificity. AUCs and cut-off points for proteins with statistically significant associations with diagnosis are presented in the results section. Receiver operating characteristic curve-based cut-off points could not be chosen for all the proteins, due to the lack of discriminatory power. For the analysis of the associations between protein expressions and clinical parameters, median values were used as cut-off points for all the proteins, with these varying from 0.00 to 100.00.

IPI values were divided into two groups, with group one including patients with IPI points from 0 to 2, and group two including IPI points from 3 to 5. The number of extranodal lesions was also divided into two groups, with group one including patients with zero or one extranodal lesions, and group two including patients with two or more extranodal lesions.

A Kruskal-Wallis test for continuous variables was performed including all three different diagnoses (sDLBCL, sCNSL and PCNSL) and a Mann-Whitney *U* test for two diagnostic groups (sCNSL and PCNSL). A chi-square test was used to determine the associations between protein expressions and clinical parameters as well as protein expressions and diagnosis (sDLBCL and sCNSL).

## Results

### Immunohistochemical expression in lymphoma samples

E-cadherin did not show any immunoreactivity. For ITGA10, CD44, PTEN, cadherin-11, N-cadherin, P-cadherin and lactoferrin, cytoplasmic immunostaining was seen with all the

antibodies used. Nuclear immunostaining was seen with all the antibodies used; except for CD44 there was no nuclear immunoreactivity. Membranous immunostaining was seen for ITGA10, CD44, PTEN and CDH12. The staining percentages are presented in Supplementary Table 2, available at *Carcinogenesis* Online. Figure 1 presents IHC staining patterns.

### Immunoelectron microscopy expression in lymphoma samples

In PCNSL samples, the strongest expressions were seen for CD44, PTEN, cadherin-11 and N-cadherin. ITGA10 membranous expression was substantial in sCNSL. CD44 membranous and CDH12 nuclear expressions were substantial in sDLBCL. Strong nuclear expression of CDH12 was also detected in lymph node hyperplasia.

The proteins studied were also detected outside of lymphoma cells. Particularly lactoferrin was seen outside of lymphoma cells in all samples, most strongly in PCNSL. In sDLBCL and lymph node hyperplasia CDH12 was similarly expressed in normal lymphocytes as in lymphoma cells. CDH12 staining was also seen outside of lymphoma cells in PCNSL and sCNSL samples. In PCNSL, CD44 seemed to be expressed on the plasma membranes of the cells that came into contact with lymphoma cells with cell protrusions. PTEN was similarly expressed in PCNSL and sCNSL samples in cells that had long cell protrusions that came into contact with lymphoma cells. It was expressed in the membranes of these cells. The precise cell type could not be determined. Possibly these were antigen-presenting microglial cells. In PCNSL samples ITGA10 was also detected in cells that were not lymphoma cells. In addition it was seen in myelinated and non-myelinated neurons. Low expression of N-cadherin was detected in PCNSL in cells surrounding lymphoma cells. In sCNSL, the expression in surrounding cells was higher. High expression of cadherin-11 was seen in the plasma membranes of cells surrounding the lymphoma cells in the sCNSL sample. These cells could possibly represent antigen-presenting microglial cells. Supplementary Figure 1, available at *Carcinogenesis* Online, presents electronmicroscopic expression in these cells.

Supplementary Table 3, available at *Carcinogenesis* Online, represents immunoelectron microscopy expression in all samples. Figure 2 presents electronmicroscopic expression.

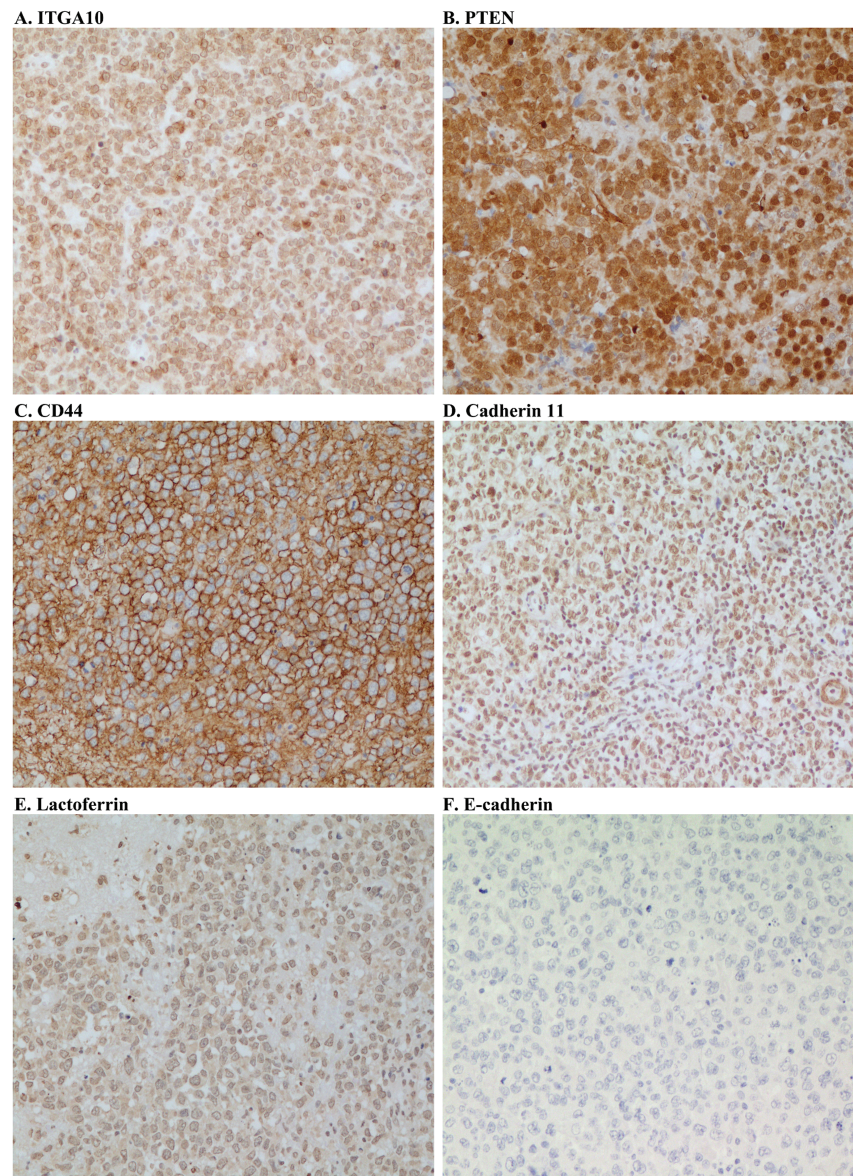
### Differences in gene-expression

The molecules studied were found in GEP data but only CD44, cadherin-11, lactoferrin and E-cadherin presented differences in the gene expression levels, being underexpressed in sCNSL compared to sDLBCL (Table 1).

### Associations with central nervous system involvement

There were statistically significant differences between the expression of ITGA10, CD44, PTEN, cadherin-11 and lactoferrin stainings in different diagnostic groups. The differences gained from the Kruskal-Wallis test for continuous variables with all three diagnoses are presented here, mean and median values representing the staining percentages. Table 2 represents the differences gained from the Mann-Whitney *U* test comparing two diagnoses; sCNSL versus PCNSL and Table 3 the results of the chi-square test with chosen cut-off points (Tables 2 and 3).

ITGA10 cytoplasmic expression was lowest in sDLBCL (mean rank = 22.62, median = 0.00), higher in sCNSL (mean rank = 33.20, median = 50.00) and highest in PCNSL (mean rank = 46.85, median = 100.00) ( $P < 0.0005$ ) (Figure 3A).



**Figure 1.** Immunohistochemical staining patterns. (A) Positive cytoplasmic and membranous staining, with some faint nuclear staining, for ITGA10 in sCNSL sample, (B) positive nuclear and cytoplasmic staining for PTEN in PCNSL sample, (C) positive membranous staining for CD44 in PCNSL sample, (D) positive nuclear and cytoplasmic staining for cadherin-11 in sDLBCL sample, (E) positive nuclear and cytoplasmic staining for lactoferrin in PCNSL sample and (F) negative staining for E-cadherin in sDLBCL sample. Magnification  $\times 20$ .

ITGA 10 membranous expression was lowest in sDLBCL (mean rank = 25.40, median 0.00), higher in PCNSL (mean rank = 26.08, median = 0.00) and highest in sCNSL (mean rank = 40.25, median = 100) ( $P = 0.002$ ) (Figure 3B).

CD44 membranous expression was lowest in sCNSL (mean rank = 34.82, median = 50.00), higher in sDLBCL (mean rank = 46.22, median = 70.00) and highest in PCNSL (mean rank = 67.54, median = 100.00) ( $P = 0.001$ ) (Figure 3D).

PTEN nuclear expression was lowest in sDLBCL (mean rank = 25.72, median = 0.00), higher in PCNSL (mean rank = 36.04, median = 0.00) and highest in sCNSL (mean rank = 38.37, median = 5.00) ( $P = 0.024$ ) (Figure 3C).

Cadherin-11 nuclear expression was lowest in sCNSL (mean rank = 34.02, median = 30.00), higher in sDLBCL (mean rank = 51.80, median = 100.00) and highest in PCNSL (mean rank = 52.23, median = 100.00) ( $P = 0.006$ ) (Figure 3E).

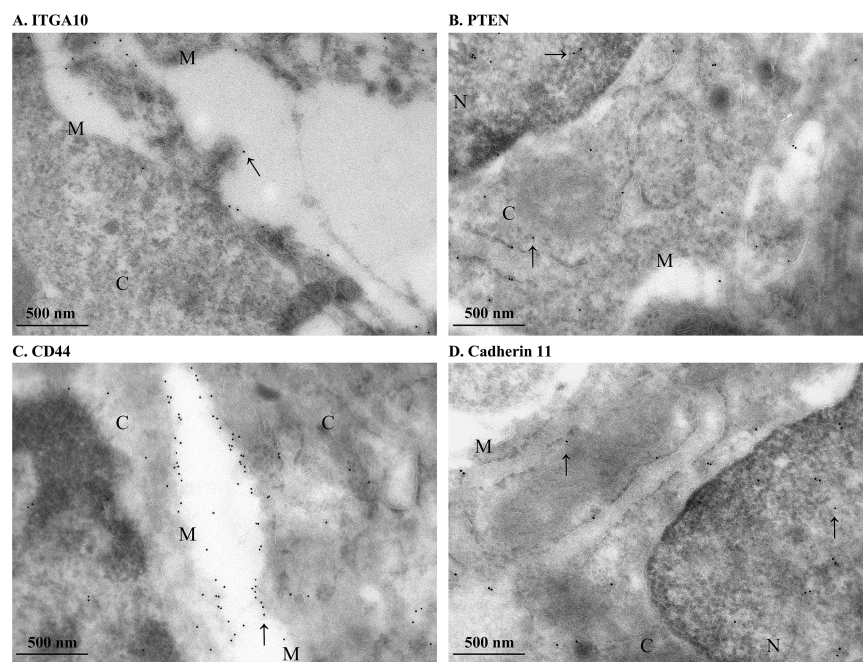
Cadherin-11 cytoplasmic expression was lowest in sCNSL (mean rank = 41.52, median 0.00), higher in sDLBCL (mean rank 46.64, median = 0.00) and highest in PCNSL (mean rank = 54.00, median 0.00) ( $P = 0.048$ ). When comparing the expression between just two groups, sCNSL and sDLBCL, the result was non-significant.

Lactoferrin cytoplasmic expression was lowest in sDLBCL (mean rank = 28.50, median = 0.00), higher in sCNSL (mean rank = 30.18, median = 0.00) and highest in PCNSL (mean rank = 52.85, median = 100.00) ( $P < 0.0005$ ) (Figure 3F). When comparing the expression between just two groups, sCNSL and sDLBCL, the result was non-significant.

### Associations with clinical parameters

CDH12 nuclear and ITGA10 membranous expressions had a positive association with the number of extranodal lesions





**Figure 2.** Micrographs representing electronmicroscopic expression patterns. (A) In the sCNSL sample ITGA10 molecules were localized to the cell membrane, (B) in the PCNSL sample PTEN molecules were mostly localized to the nucleus and cytoplasm, only few molecules seen on the cell membrane, (C) in the sDLBCL sample CD44 molecules were mainly located to the cell membrane, with few molecules seen in the cytoplasm, (D) in the PCNSL sample cadherin-11 molecules were localized to the nucleus and cytoplasm. In all of the images the cells are malignant lymphocytes. N = nucleus, C = cytoplasm, M = cell membrane, ↑ marks one of the molecules. Magnification  $\times 46\,000$ .

**Table 1.** Gene-expression profiling results

Protein	Fold-change
ITGA10	NC
CD44	-3.93
PTEN	NC
Cadherin-11	-42.04
CDH12	NC
N-cadherin	NC
P-cadherin	NC
Lactoferrin	-1200.21
E-cadherin	-16.90

Negative fold-changes mark decreased expression in sCNSL compared to sDLBCL. The fold change for CD44 is a mean of five different values from five different probes. NC, no change.

( $P = 0.026$  and  $P = 0.006$ , respectively). CD44 membranous expression had an inverse association with the number of extranodal lesions ( $P = 0.037$ ). CD12 nuclear, lactoferrin cytoplasmic, ITGA10 cytoplasmic and ITGA membranous expressions had a positive association with the IPI score ( $P = 0.010$ ,  $P = 0.029$ ,  $P = 0.022$  and  $P = 0.007$ , respectively). PTEN nuclear expression had a positive association with lactate dehydrogenase ( $P = 0.002$ ). Higher CD44 membranous expression was associated with the non-GC phenotype ( $P = 0.049$ ). CD44 membranous expression also had a positive association with age over 60 ( $P = 0.034$ ).

## Discussion

In this work, we studied the biology of DLBCL's CNS tropism with IHC, IEM and GEP with the aim of finding biological markers that would define DLBCL patients at high risk of CNS recurrence. We found that elevated levels of ITGA10 and PTEN were associated

with CNS tropism, while CD44 and cadherin-11 expressions seemed to be protective of secondary CNS disease. Interestingly though, CD44, cadherin-11 and lactoferrin expressions were highest in PCNSL samples.

CNS recurrence occurs in about 5% of systemic DLBCL patients (2,3). It is a difficult complication with a very poor prognosis. Abramson et al. conducted a retrospective study with results that were later confirmed in a prospective CRY study by Nordic Lymphoma Group, showing that CNS relapse risk can be reduced with high-dose methotrexate containing prophylactic chemotherapy (6,27). This conclusion was further supported by a retrospective study by Ferreri et al (28). However, because high-dose methotrexate is a resource-demanding therapy with considerable toxicity, it should be limited to patients with a high risk of CNS recurrence. Currently identification of these high-risk patients is performed based on clinical factors, which unfortunately have a poor specificity (8). Thus there is a need to define biological markers with a higher specificity for separating patients in need of CNS prophylactic treatment. Currently there are suggestions that double-hit genotype, non-GC phenotype, as well as CD5 positivity may have some prognostic relevance in this matter (29–31).

Understanding the pathophysiology of CNS lymphomas has been a challenge for years. An attractive hypothesis is that these malignant cells originate from extracranial sites but end up homing into the CNS due to highly selective CNS tropism. Results from recent studies have supported this hypothesis (32). In studies with GEP, differences in transcription of several proteins have been observed when comparing normal lymphatic tissue and nodal or extranodal DLBCL with PCNSL. Our results are in line with these previous findings because it seems that adhesion-, migration- and ECM-related molecules play a pivotal role in CNS involvement of DLBCL (33,34).

In our previous study of CNS lymphomas, we assumed that CXCR4/CXCL12 and CXCR5/CXCL13 axis play an important role



**Table 2.** Associations of protein immunohistochemical expressions and diagnosis (sCNSL and PCNSL)

Protein, location	Mean rank, sCNSL	Median sCNSL	Mean rank, PCNSL	Median PCNSL	P value
CD44, M	18.12	50	30.96	100	0.002
Lactoferrin, C	18.00	0	33.23	100	<0.001
P cadherin, N	19.84	5	28.85	20	0.030
ITGA10, C	18.70	50	29.62	100	0.006
ITGA10, M	24.73	100	15.69	0	0.014

C, cytoplasm; M, membrane; N, nucleus.

**Table 3.** Cut-off points and associations of protein IHC expressions and secondary CNS disease

Protein, location	AUC	CI	Cut-off	Association	P value
CD44, M	0.668	0.514–0.821	70.00	↓	0.015
PTEN, N	0.694	0.547–0.840	0.00	↑	0.011
ITGA10, N	0.657	0.500–0.815	10.00	↓	0.044
ITGA10, C	0.679	0.519–0.838	10.00	↑	0.002
ITGA10, M	0.745	0.606–0.884	0.00	↑	0.007
Cadherin-11, N	0.673	0.514–0.831	70.00	↓	<0.001

Diagnoses studied, sDLBCL and sCNSL. AUC, area under curve; CI, confidence interval; C, cytoplasm; M, membrane; N, nucleus. Cut-off signifies the chosen cut-off point. ↓ = inverse association and ↑ = positive association with CNS involvement.

in CNS tropism of DLBCL (9). Concordantly Rubenstein et al. (35) have shown that CXCL12 and CXCL13 act as mediators of CNS lymphoma cell chemotaxis. In line with these findings, CXCR4/CXCL12 signalling has been shown to induce EMT or an EMT-like process that enables cell migration towards the expression of chemokine ligands and invasive abilities (36–39). CXCR4/CXCL12 signalling has also been shown to induce integrin and CD44 activation and also CD44 localization to the leading edge during migration in human CD34+ stem/progenitor cells. These events together with integrin expression facilitate cell attachment to the extracellular space as well as the invasion and mobilization of cells (10,12). CD44 affects human haematopoietic progenitor cell trafficking to the BM via interactions with hyaluronic acid (10). It is noteworthy that hyaluronic acid is also the main component of brain extracellular matrix (40). He et al. (41) found that perivascular tumour cells in PCNSL showed a strong CD44 expression explaining the perivascular staining pattern. Our results are in line with this data describing the highest CD44 expression in PCNSLs.

Our findings of integrin expression were in line with the hypothesis of cell migration, as ITGA10 showed a strong CNS tropism favouring function (42,43). The expression of membranous ITGA10 had a positive association with two of the known clinical risk factors for CNS tropism: the number of extranodal lesions and higher IPI score.

PTEN is generally regarded as a tumour suppressor gene and in cancers its loss is associated with increased proliferation rate (44). Loss of PTEN expression has also been shown to occur in some B-cell lymphomas (45,46). We found its nuclear location to be associated with enhanced CNS tropism. In a work by Goh et al. nuclear trafficking of PTEN after an injury to the neurons was shown to lead to survival and thus to protect these cells from cell death, probably due to the depletion of PTEN from its functional place, the cytoplasm and cell membrane (47). Loss of PTEN has been shown to result in increased CXCR4 and CXCL12 expression and invasion in prostate cancer cells (11). If nuclear localization is able to inhibit the cell cycle regulatory functions of PTEN, it could be that it can also lead to increased CXCR4 expression as seen when PTEN expression is lost, and enhance

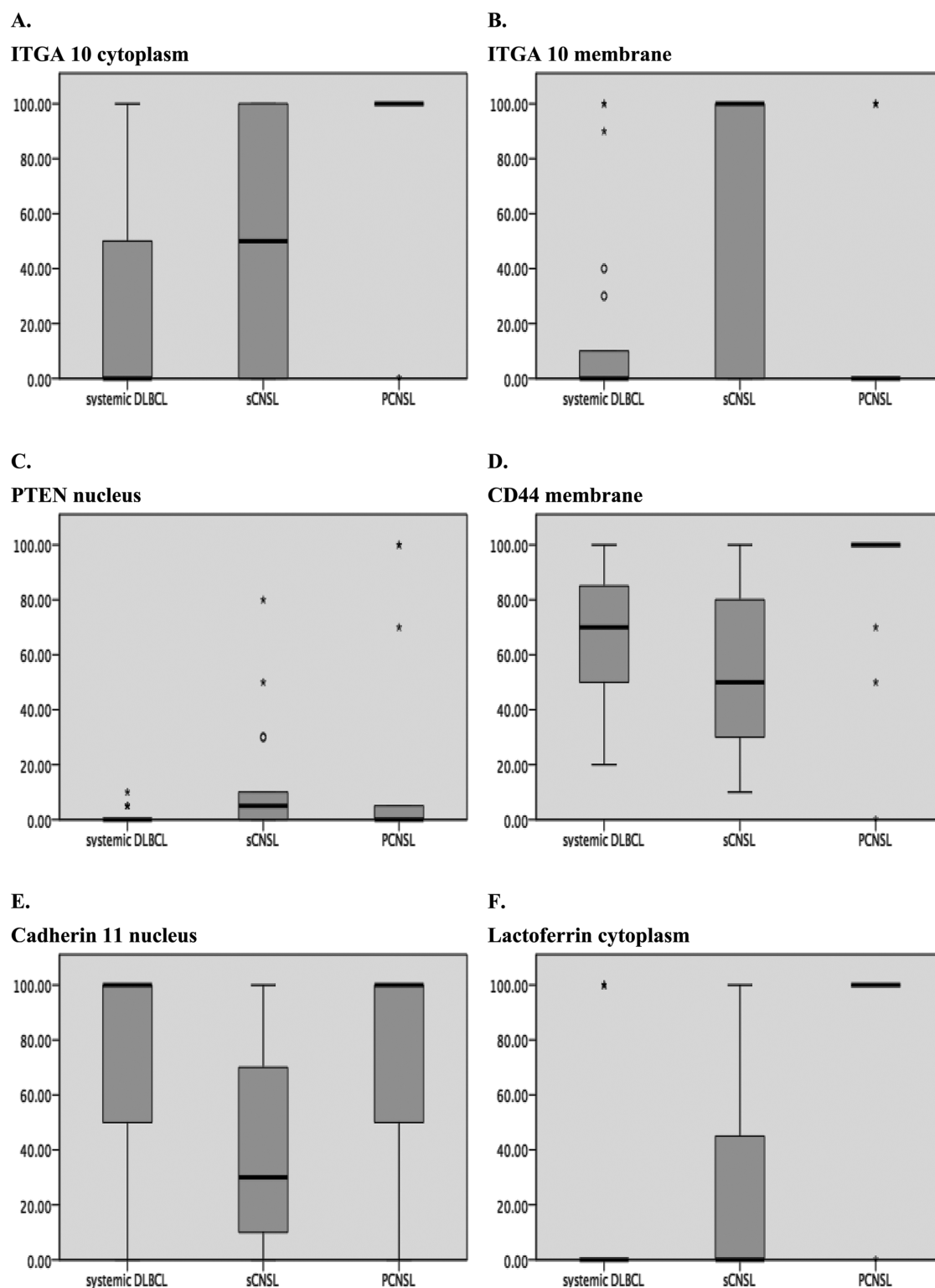
the CNS tropism in this way. In our study nuclear PTEN was the only protein associated with increased lactate dehydrogenase levels.

Cadherin-11 cytoplasmic and nuclear expressions were highest in PCNSL, yet the latter was associated inversely with sCNSL. In contrast to this, in epithelial cancers, cadherin-11 is often associated with mesenchymal features gained through EMT (17).

In our results, lactoferrin expression was highest in PCNSL and second highest in sCNSL when studied with IHC and IEM. When comparing sDLBCL and sCNSL, no statistically significant differences were seen. In GEP, higher expression level was seen in sDLBCL samples but GEP did not include PCNSL samples that had the most abundant expression in IHC and IEM. Lactoferrin has been shown to be produced in the CNS by activated microglial cells and its expression has been associated with neurological disorders affecting the CNS, such as Alzheimer's and Parkinson's diseases. It seems that lactoferrin expression is upregulated as a consequence of inflammatory conditions and under oxidative stress (48). Most likely this could also be the case in PCNSL.

Taken together, our findings support the hypothesis that CXCR4 signalling might participate in the initiation of lymphoma cells homing into the CNS. This again may enable the morphological and expressional changes seen in this study. In a work by Ricciardi et al. (49) mesenchymal stromal cell-like features gained through EMT conferred cancer cells with immunomodulatory properties which again possibly attenuate cancer cell immune escape and progression.

When the IHC, IEM and GEP results were compared they were mostly in concordance with each other. All of the molecules studied were found in GEP and four presented with gene-expressional changes. The expressional changes were similar to IHC and IEM results, except that E-cadherin was not seen to be expressed at a protein level. Lactoferrin expression also presented with minor differences. The small number of GEP samples used in this study limits the reliability of the results. However, concordant results seen in larger IHC and IEM materials indicate that these findings seem to present a relevant event



**Figure 3.** Associations of protein immunohistochemical expressions and diagnoses. A Kruskal-Wallis test was used to determine the associations between protein expression and different diagnoses for (A) ITGA10 cytoplasmic expression ( $P < 0.0005$ ), (B) ITGA10 membranous expression ( $P = 0.002$ ), (C) PTEN nuclear expression ( $P = 0.024$ ), (D) CD44 membranous expression ( $P = 0.001$ ), (E) cadherin-11 nuclear expression ( $P = 0.006$ ) and (F) lactoferrin cytoplasmic expression ( $P < 0.0005$ ). The boxes represent the interquartile range, the whiskers represent the 10th and 90th percentiles and the highlighted lines across the boxes indicate the median values. Circles represent extremes and asterisks represent outliers.

in DLBCL CNS tropism. The few discrepancies may be explained by the methods used; IHC measures the expression of the studied proteins within a sample tissue whereas GEP measures

the expression of specific messenger RNAs in the sample. IHC and IEM results were also mostly in concordance with each other, with only a couple of differences. IHC results represent

a collection of multiple samples and a large number of cells are evaluated. IEM expression on the other hand represents one or two samples, and from those a single cell per view. This might cause a selection bias if the expression in this specific sample is below average. Using light microscopy, it is harder to tell cytoplasmic and membranous staining apart than it is in electron microscopy. This might represent another bias to the results.

Our findings imply that some biological events are common in both primary and secondary CNS lymphomas. Yet the fact that PCNSL samples are brain biopsies and sCNSL samples from lymph nodes from a time of the primary diagnosis, the differences in the microenvironment might also lead to the seemingly different results observed between these two groups, and this impact could not be excluded in this study. In addition, PCNSL and sCNSL are two different entities and there might also be differences due to this.

In this work, we have shown that lymphoma CNS tropism is linked to enhanced expression of integrins (ITGA10 in this study) and PTEN, when again sCNSL is linked to decreased CD44 and cadherin-11 expressions. In PCNSL, lactoferrin seems to be upregulated. This data is in line with current knowledge of morphological and protein expressional changes occurring during cell migration and invasion, and with the current GEP data of CNS lymphomas where differences in the expression of adhesion and ECM-related genes have been seen (33,34). These EMT-like changes seen in our study might also attenuate lymphoma cell immune escape (49). To our knowledge, this is the first study exploring the role of ITGA10, CD44, PTEN, cadherin-11, CDH12, N-cadherin, P-cadherin, lactoferrin and E-cadherin in DLBCLs and especially in CNS lymphomas. The present study was a retrospective one and the size was too small to calculate the positive and negative prognostic values of these markers as well as to perform multivariate analyses comparing independent values of these markers with clinical factors. Currently the most powerful markers for prediction of CNS tropism seem to be non-GC phenotype, double-hit genotype and CD5 positivity (28–31). From our candidate markers, only CD44 expression was associated with non-GC phenotype, implying that the rest of the proteins studied may give new prognostic value for CNS relapse prediction in both GC and non-GC phenotypes. Unfortunately translocation data was not available from our cases. However the findings are able to support a hypothesis that cytoplasmic and membranous ITGA10, nuclear PTEN and membranous CD44 expressions should be studied as promising candidates to be used as biological markers for screening of systemic DLBCL patients at elevated CNS relapse risk. These results are highly preliminary and need to be validated in a larger prospective trial.

## Supplementary material

Supplementary material is available at *Carcinogenesis* online.

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